Osteoarthritis and Cartilage (2010) **18,** 397–405

doi:10.1016/j.joca.2009.09.011

Osteoarthritis and Cartilage

Collagen fibril disruption occurs early in primary guinea pig knee osteoarthritis

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Summary

Objective: A major barrier inhibiting the discovery of structural modifying agents for osteoarthritis (OA) is an incomplete understanding of early disease events. Herein, we investigated the time course of collagen II cleavage and fibril disruption in the well-validated Hartley guinea pig model of spontaneous OA of the knee.

Methods: Knee joints of 46 male Hartley guinea pigs were analyzed at 3 weeks, 2, 4, 7, 10, 12, and 18 months of age for histological severity of OA, cartilage collagen fibril disruption by semi-quantitative polarized light microscopy, and expression of type II collagen degradation biomarkers, 9A4 and Coll2-1, by immunohistochemistry. In addition, serum biomarkers specific for collagen II degradation, CTX-II, C2C, and Coll2-1 were quantified.

Results: Collagen fibril disruption and expression of the collagenase-generated cleavage neoepitope, 9A4, were observed as early as 2 months of age, despite the appearance of histological OA at 4 months of age. Only serum Coll2-1 increased coincident with the early disruption of the collagen fibril between 3 weeks and 7 months, in contrast to serum C2C, which did not change significantly or correlate with histological severity. Inversely, CTX-II declined dramatically from 3 weeks to 4 months and remaining low thereafter, coincident with growth plate turnover.

Conclusions: Collagenase cleavage and disruption of the type II collagen network are early OA disease events in this model, preceding histological evidence of proteoglycan loss. The markedly different serum profiles of collagen II-related biomarkers during the early stages of disease development suggest compartmental segregation and temporal regulation of collagen degrading enzymes.

Key words: Osteoarthritis, Collagen, Biomarkers, Animal model, Guinea pig.

Introduction

A greater focus on early, pre-radiographic osteoarthritis (OA) is needed to provide insight into targets for intervention at a stage more amenable to modification. The early events are difficult to characterize in primary human OA due to the great uncertainty surrounding disease onset and the great biological variability in disease progression. The Hartley guinea pig however provides a consistent model of spontaneous and progressive degeneration of the knee that closely resembles knee OA in humans[1,2]. This animal model is especially attractive for studies of OA pathology because it represents primary idiopathic OA, and the guinea pig, unlike rodents, expresses both interstitial collagenases-1 and -3 which are associated with cartilage damage[3], and OA in humans[4]. We hypothesized that the Hartley guinea pig would provide a physiologically relevant model in which to analyze early OA disease events to gain insights into mediators of disease onset. This study focused on characterizing the collagen II-related events relative to histological OA and proteoglycan loss in the course of disease development.

The collagen fibril network maintains the volume, shape, and tensile strength of the extracellular matrix of articular cartilage. Type II collagen is relatively specific to articular cartilage and is the most abundant cartilage protein, representing 15–25% of the wet weight, 50% of the dry weight[5], and >90% of the total collagen content[6]. Disruption of this fibrillar network is mediated by specific matrix metalloproteinase (MMP) enzymes that cleave collagen, namely collagenases-1, -2 and -3 (MMPs-1, -8, and -13). Collagenase-mediated cleavage of type II collagen produces two fragments: a 3/4 length fragment and a ¼ length fragment. Once this initial cleavage has taken place, the triple helical collagen fibril unwinds and becomes susceptible to degradation by other enzymes such as gelatinases. The biological half-life of type II collagen is estimated to be 117 years in human adult cartilage[7], contributing to the concept that collagen fibril degradation is a key feature of OA because the irreversible damage imposed on this matrix molecule is not adequately compensated for by new protein synthesis.

The proteolysis of type II collagen yields three types of neoepitope fragments accessible to body fluids[8]: the collagenase-generated C2C neoepitope on the carboxy end of

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Received 14 May 2009; revision accepted 27 September 2009.

the 3/4 fragment[9], the denaturation-generated Coll2-1 neoepitope[10] in the triple helical domain near the carboxy end of the 3/4 fragment, and the matrix metalloprotease-generated neoepitope[11,12] CTX-II[13], localized to the mature carboxy end or telopeptide of the collagen molecule (Fig. 1). We have found that all three types of collagen II neoepitopes are measurable in the guinea pig, providing a means of quantifying the time course of collagen II metabolism in this animal model. These provided adjunctive measures with which to monitor the disease course to complement the primary histological, and polarized light microscopic outcomes of collagen degradation in this model system.

Methods

ANIMALS

Six male Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA) at 3 weeks of age, and an additional forty male Hartley guinea pigs were obtained at 2 months of age and raised until sacrifice at 2 ($n=6$), 4 ($n=6$), 7 ($n=6$), 10 ($n=6$), 12 ($n=6$), and 18 ($n=10$) months of age. The Institutional Animal Care and Use Committee approved all procedures. Blood was collected into SST Vacutainer tubes (VWR International, West Chester, PA) at each time point. The samples were centrifuged at 3000 rpm for 15 min and sera were aliquoted and frozen at −80 °C until analyzed. The right knee joint from each animal was fixed for 24 h in 10% buffered formalin, followed by decalcification in 10% ethylenediaminetetraacetic acid (EDTA) in 0.1 M phosphate buffer, pH 7.6–7.8. Paraffin sections (5 μM) of the central region of the joint were stained with either toluidine blue for histological analyses or picrosirius red and examined under polarized light to evaluate collagen birefringence.

HISTOLOGICAL GRADING

A semi-quantitative modified Mankin histological grading system described previously[14] was used to evaluate OA severity in each tibial plateau and femoral condyle. This scoring scheme permits site-specific and separate scoring of cartilage structure (extent and severity of surface irregularities including fibrillation and clefts [0–8]), and proteoglycan content (as determined by extent of toluidine blue staining [0–6]) and has demonstrated a correlation with severity of OA as reflected by the synovial fluid biomarkers, cartilage oligomeric matrix protein (COMP) and keratan sulfate (KS)[14–16]. Histological sections were graded by two observers (JLH, VBK) blinded to the animal status.

POLARIZED LIGHT MICROSCOPIC ANALYSES

Slides containing histological sections for analyses of collagen network orientation were exhaustively deparaffinized, as paraffin is strongly birefringent. Sections were hydrated as described previously[17] and treated at 37 °C for 18 h in 2.0 mg bovine testicular hyaluronidase in 1.0 ml 0.1 M phosphate buffer at pH 6.0 to remove chondroitin sulfate molecules[18], to eliminate possible masking of collagen's cationic binding sites for the polyanionic

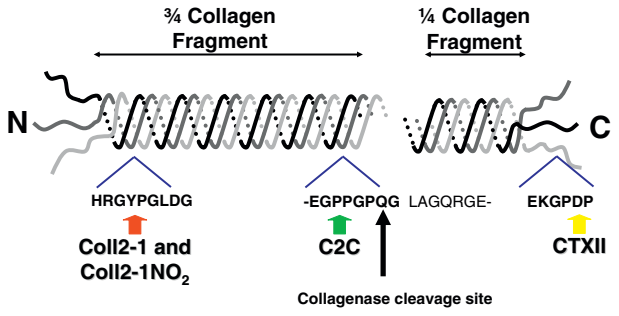

Fig. 1. Schematic representation of the specific type II collagen epitopes measured. The epitopes measured in sera result from the three types of collagen degradation: collagenase cleavage (C2C), denaturation (Coll2-1), and MMP-generated collagen telopeptides (CTX-II).

Sirius red molecules[19]. Sections were then stained for 30 min in 0.1% Sirius red F3B (Polysciences, USA) dissolved in saturated picric acid, which enhances the normal birefringency of collagen fibers in tissue sections[20], and then washed, dehydrated, and mounted with cover slips. Sections were analyzed with a Nikon microscope equipped with polarizing filters and the induced birefringence was determined by turning the analyzer in two opposite directions. The optical properties of the extracellular matrix, namely the presence or absence of birefringence, indicated the orientation of the collagen fibers[20,21] of the articular cartilage. The relative loss of collagen birefringence (expressed as a percentage) was based on a comparison with the pattern of birefringence in the 3-week old guinea pig knee cartilage which was taken to represent normal (100%) birefringence. These assessments were made blinded to animal age and histological score. The images were obtained using Metamorph Software 4.01™. The entire area of articular cartilage was circumscribed to obtain a total area. Likewise, areas of normal vs altered birefringence were circumscribed allowing expression of birefringence results as percentages of total cartilage area.

IMMUNOHISTOCHEMISTRY

Sections of guinea pig knees were stained with monoclonal antibody (mAb) 9A4 (kindly provided by Dr. Peter Mitchell) and antisera D3 containing antibodies to Coll2-1. The 9A4 mAb recognizes the collagenase-generated neoepitope at the C-terminus of the collagen 3/4 fragment[22] and was readily available whereas the anti-C2C antibody was not commercially available and has been reported to be unsuitable for immunohistochemistry[23]. The antiserum to Coll2-1 recognizes a denaturation epitope of collagen II that results from unwinding and thus detects only degraded collagen, ether *in situ* (in cartilage) or in body fluids[24]. Paraffin-embedded sections were deparaffinized with xylene and then rehydrated with graded ethanol. Endogenous peroxidase activity was blocked by incubation of the sections with freshly prepared 0.5% (vol/vol) H_2O_2 in absolute ethanol for 10 min at room temperature. To enhance the permeability of the extracellular matrix, glycosaminoglycans were removed by incubating the sections with 0.4 U/l proteinase-free chondroitinase ABC (Sigma) in 0.1 M Tris–HCl pH 8.0 for 30 min at 37 °C. Non-specific binding was blocked by incubation of the sections with 100 μl of 1% normal goat serum (Jackson ImmunoResearch) diluted in tris buffered saline (TBS); 50 mM Tris, 138 mM NaCl pH 7.6 for 30 min. For staining with mAb 9A4, sections were incubated overnight at 4 °C with 15 μg/ml of mAb 9A4 according to previously described methods[3]. Staining for Coll2-1 was performed by incubating sections for 2 h with 100 μl of antisera derived from rabbits (D3) diluted 1/100 in TBS containing 1% normal goat serum, as described previously[23]. As a negative control, sections were treated with goat non-immune serum diluted 1/100 as the substitute for the primary antibody.

COLLAGEN II BIOMARKER ANALYSES

Levels of serum C2C were quantified by a commercially available enzyme-linked immunosorbent assay (ELISA) (IBEX, Montreal, Quebec) in serum diluted 1:2, as per the manufacturers' protocol. The intra- and inter-assay variability for C2C was 3.3% and 16.3% respectively, as reported previously[16]. The denaturation epitope, Coll2-1 and the nitrosylated epitope, Coll2-1NO_2, localized to the helical domain of type II collagen[24,25] were quantified in guinea pig sera (diluted 8-fold) by competitive ELISA in duplicate with polyclonal rabbit antisera (D3 and D37, respectively)[24]. For Coll2-1, the intra-assay and inter-assay variability were 8.2% and 9.3% respectively. Levels of serum CTX-II were quantified using the serum Preclinical Cartilaps ELISA (Nordic Bioscience), an assay designed to detect degradation products of C-terminal telopeptides of type II collagen in animal sera. The intra-assay and inter-assay variability were 5.1% and 8.9% respectively. Biomarker assays were performed blinded to guinea pig age or histological data. Results were expressed as mean + standard deviation (SD).

STATISTICAL ANALYSES

All statistical analyses were performed using Prism GraphPad 5.0 (GraphPad Software, La Jolla, CA). The individual components of the histological scores (cartilage structure and proteoglycan content) and the birefringence of the four joint surfaces [medial and lateral femoral (LF) condyles, medial and lateral tibial (LT) plateaus] at the various ages were analyzed by the one-way analysis of variance (ANOVA), non-parametric Kruskal–Wallis test, followed by Dunn's Multiple Comparison post-hoc test; the non-parametric Mann–Whitney test was used to compare the differences in histological scores and measures of birefringence between either medial or lateral compartments or tibial and femoral surfaces, as well as the levels of Coll2-1 at 3 weeks and 4 months of age. To evaluate for potential correlation of the biomarkers, non-parametric Spearman correlations were calculated. In addition, total histological scores were separated into quartiles and corresponding levels of biomarkers were analyzed by one-way ANOVA, non-parametric Kruskal–Wallis

test, followed by Dunn's Multiple Comparison post-hoc test. Data were considered statistically significant at $P < 0.05$.

Results

HISTOLOGICAL SCORING AND COLLAGEN BIREFRINGENCE

Histological changes in cartilage structure and/or proteoglycan content consistent with histological knee OA were apparent by 4 months of age at all four surfaces of the knee. The greatest severity of both cartilage structural damage and loss of proteoglycan was evident for the medial tibial (MT) surface of the knee and worsened throughout the course of disease development, with the most pronounced change in histological scores occurring between 4 and 7 months of age (Fig. 2). Significant differences in cartilage structure scores were observed between medial and lateral compartments (7 month $P = 0.05$; 10 month $P = 0.02$; 12 month $P = 0.03$; 18 month $P = 0.0002$) as well as between tibial and femoral surfaces at time points from 7 to 18 months of age (7 month $P = 0.05$; 10 month $P = 0.10$; 12 month $P = 0.01$; 18 month $P = 0.03$). In contrast, differences in proteoglycan scores were only significant between tibial and femoral surfaces at time points from 7 to 12 months of age (7 month $P = 0.002$; 10 month $P = 0.01$; 12 month $P = 0.09$; 18 month $P = 0.005$). Comparing the medial and lateral compartments, there were no significant differences in proteoglycan content scores with the exception of the 18 month time point (18 month $P = 0.04$).

The tibial surface at 3 weeks of age showed a typical pattern of birefringence: normal birefringence on the surface zone, no birefringence in the middle zone, and normal birefringence in the deep zone. Representative sections of 2, 4, 7, 10 and 12-month-old guinea pig knee joints, depicting examples of altered cartilage birefringence, are shown in Fig. 3. Beginning at 2 months of age, the normal pattern of birefringence was diminished by a mean 14% followed by a progressive loss of normal collagen birefringence of all four surfaces with age (Fig. 4). Overall, the birefringent area of cartilage declined a mean 50% from 3 weeks to 12 months of age. From 4 months onward, reductions in normal birefringence were significantly greater on the tibial side of the joint compared with the corresponding femoral surface. However, the extent of the loss of birefringence was not significantly different between the medial and lateral compartments of the tibial plateau at any time point.

IMMUNOHISTOCHEMICAL STAINING

Immunostaining with the 9A4 mAb was performed to evaluate the time course of collagenase activity manifested as the appearance of the 3/4 collagen cleavage neoepitope. Collagen cleavage by collagenase preceded histological OA, with readily apparent 9A4 immunostaining at 2 months of age in the MT compartment (Fig. 5), compared to the uniform staining of the remainder of the full thickness articular cartilage in the LT compartment. The manifestation of collagen cleavage by collagenase (9A4 staining) detected in both the medial and LT compartments of the knee at 2 months of age corresponded to temporal alterations in birefringence and also preceded histological OA. Throughout the time course, the pattern of 9A4 immunostaining in the lateral compartment was positive and comparable to the 2-month old section; however, the pattern of 9A4 staining of the MT compartment varied in conjunction with the development of histological changes of OA. Compared to the pattern and intensity of 9A4 immunostaining at 2 months of age, at 4-months of age the MT compartment revealed more intense staining of the extracellular matrix as well as more pericellular staining of the chondrocytes in the middle zone. The staining in the 7-month-old sections was more intense than in the 4-month-old sections, specifically at the lesions sites, and there were many clusters of chondrocytes

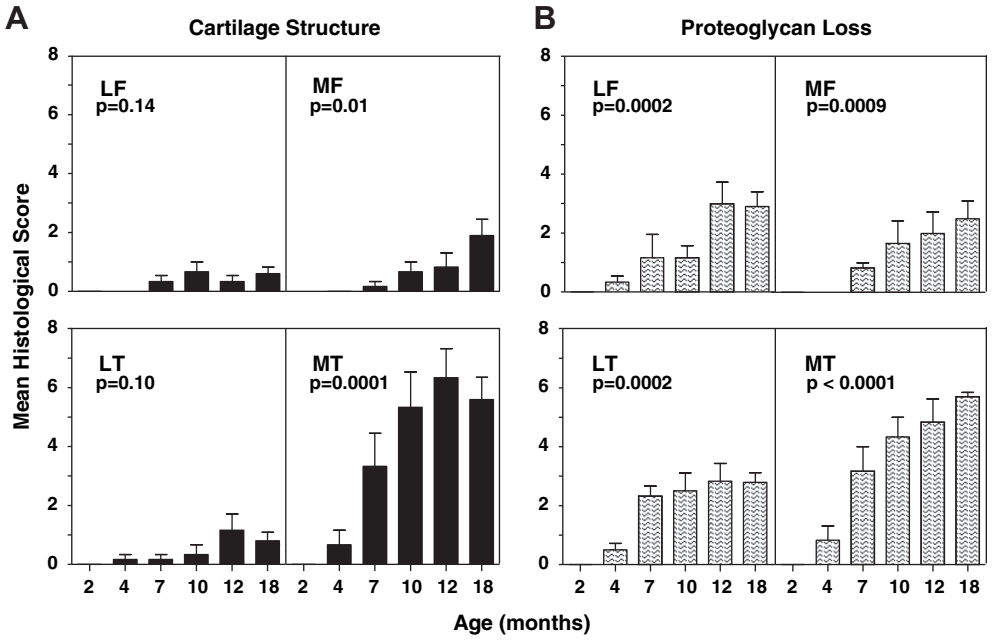

Fig. 2. Histological damage of the guinea pig knee joints by compartment. (A) Mean cartilage structure scores and (B) mean proteoglycan loss in each of the compartments (LF; LT; MF and MT) of the guinea pig knee from 2 to 18 months of age. Differences in histological scores over time at various sites within the joint were calculated by the one-way ANOVA non-parametric Kruskal–Wallis test. P values < 0.05 are considered significant.

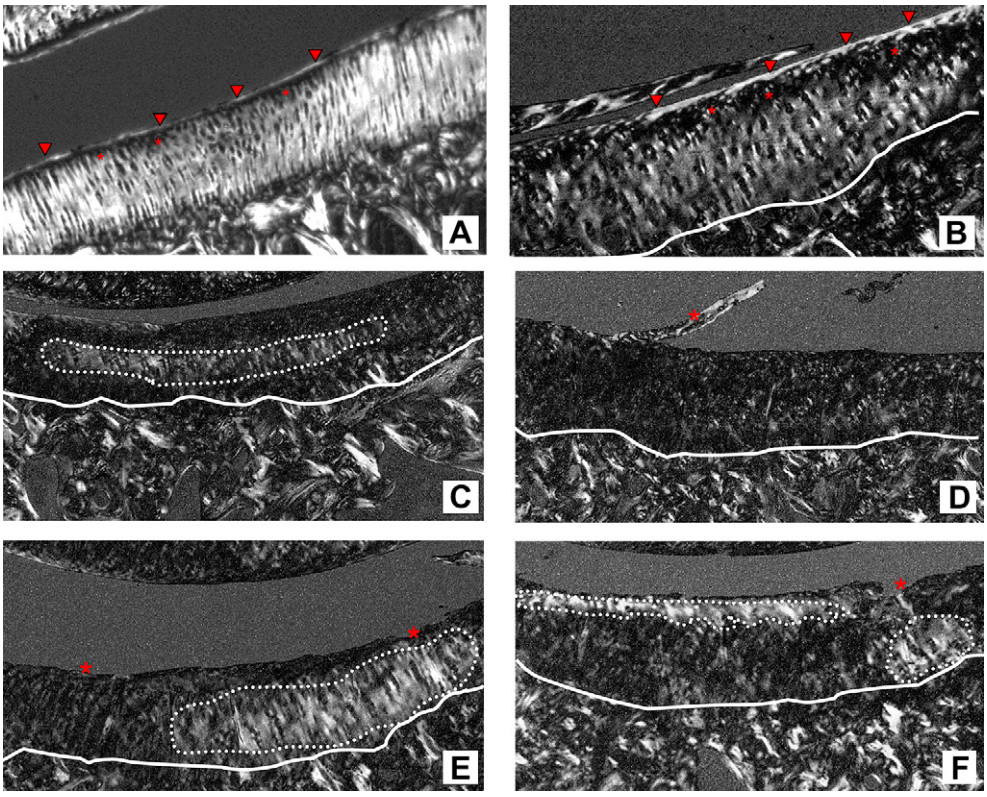

Fig. 3. Representative coronal sections of whole joints depicting variations in percent areas of normal birefringence in the tibial compartment. Appearance of the tibial surface at 3 weeks (A) showing a typical pattern of birefringence; normal birefringence on the surface (zone 1; red arrowheads), no birefringence in zone 2 (red asterisks) and normal birefringence in zone 3; at 2 months of age (B) there is normal birefringence on the surface (zone 1; red arrowheads), no birefringence in zone 2 (red asterisks) with some reduction of normal birefringence in zone 3; at 4 months of age (C) there is a loss of normal birefringence on the surface (zone 1), no birefringence in zone 2 and a mixture of normal birefringence (dashed circle) and loss of normal birefringence is noted in zone 3; at 7 months of age (D) there is loss of normal birefringence on the surface (zone 1), surface disruption (red asterisk), no birefringence in zone 2, and loss of normal birefringence is noted in zone 3; at 10 months of age (E) there is loss of normal birefringence on the surface (zone 1) and evidence of surface disruptions (red asterisks), no birefringence in zone 2, and a mixture of normal birefringence (dashed circle) and loss of birefringence is noted in zone 3; at 12 months of age (F) there is a complete loss of normal birefringence on the surface (zone 1) followed by birefringence in zone 2 (dashed circle) and again a mixture of normal (white dashed circle) and a loss of normal birefringence in zone 3. The solid white line approximates the cartilage bone interface. Original magnification 40x.

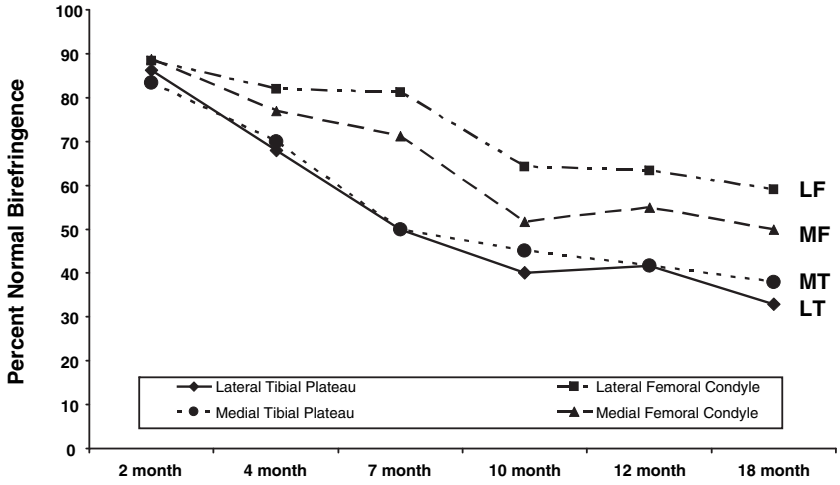

Fig. 4. Disruption of the collagen network integrity with age. Collagen birefringence was scored semi-quantitatively from picrosirius red stained sections of Hartley knee joints, aged 2 months to 18 months. The % loss of normal birefringence was estimated based upon comparison with normal birefringence as seen in a reference area. Percentage loss of birefringence for each of the four compartments is shown (LF: -■-; LT: -●-; MF: -▲- and MT: -◆-).

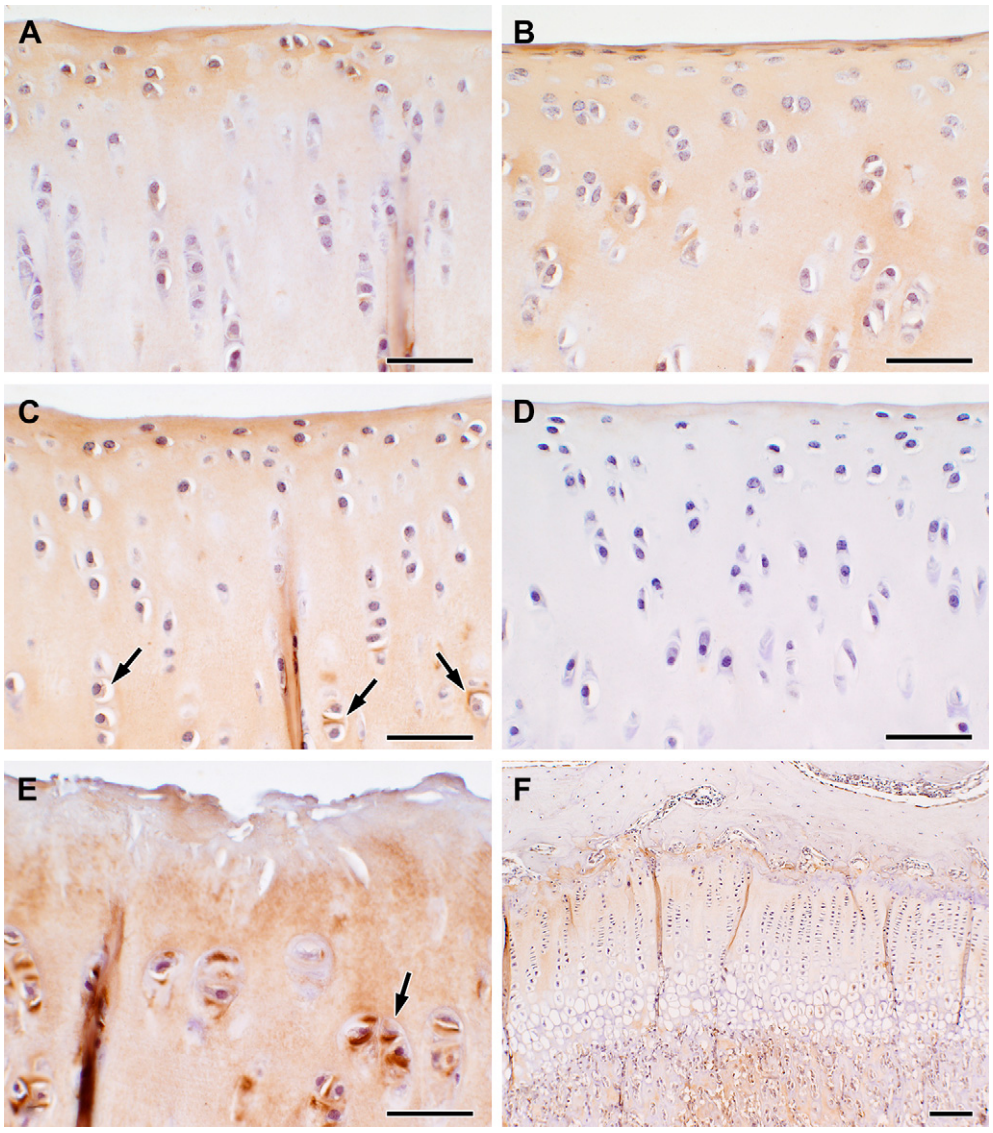

Fig. 5. Immunohistochemical expression of collagenase-generated collagen degradation. Immunostaining of representative guinea pig knee sections with monoclonal antibody (mAb) 9A4. The MT compartment (A) of a 2-month-old guinea pig depicts cleavage by collagenase in the superficial zone of the compartment which preceded histological changes associated with OA. In contrast, there is more uniform extracellular staining throughout the articular cartilage of the LT compartment at 2 months of age (B). MT compartment of 4 month old (C) and 7 month old (E) stained with monoclonal antibody 9A4 show more intense staining of the superficial zone and areas of cartilage degradation, respectively. Arrows indicate the pericellular staining of chondrocytes throughout the middle and deep zones of the articular cartilage. MT compartment of a 2-month-old guinea pig knee stained with secondary antibody only (negative control) (D). Extracellular staining of the epiphyseal plate was evident (F). Magnification bars: (A–E; $40\times = 50$ μM and F; $10\times = 100$ μM).

in the middle zone across the entire compartment that had definite pericellular staining. Extracellular staining of the epiphyseal plate was observed.

Tissue expression of Coll2-1 was quite different than that of 9A4. Instead of the predominant extracellular staining of the full thickness articular cartilage, staining of sections with polyclonal antisera to Coll2-1 (Fig. 6) revealed specific staining primarily in the chondrocytes of the superficial zone of 2-month-old guinea pig knee sections with some additional staining in the chondrocytes of the middle and deep zones of the articular cartilage and no staining of the extracellular matrix. In the 4-month-old sections, the superficial zone was devoid of chondrocytes however, staining was observed in the chondrocytes of the middle and deep zones. By 7 months of age, there was faint staining present at the surface of the articular cartilage in the areas of cartilage fibrillation and fissures, however, the superficial zone was devoid of chondrocytes, and there was less staining of the chondrocytes in the middle and deep zones, compared to that observed in the 4-month-old sections. Interestingly, there was no intra- or extracellular staining of Coll2-1 in the growth plate.

TYPE II COLLAGEN BIOMARKERS

We compared serum levels of three type II collagen biomarkers, representing three types of neoepitopes created during collagen fibril degradation (Fig. 7). Mean serum

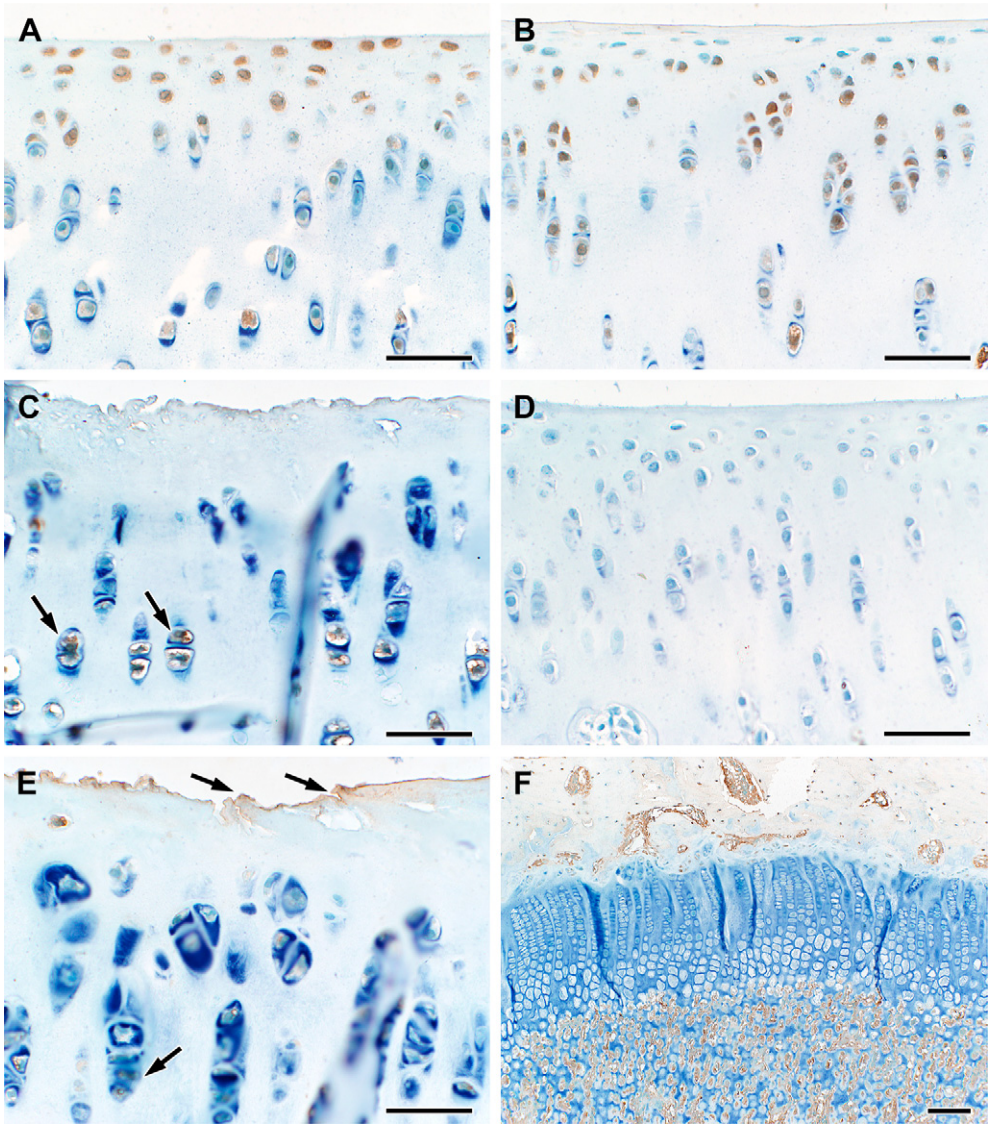

Fig. 6. Immunohistochemical expression of Coll2-1 in guinea pig knee sections. The MT (A) and LT (B) compartments of a 2-month-old guinea pig demonstrates the presence of the Coll2-1 epitope intracellularly in chondrocytes of the superficial, middle, and deep zones, but no extracellular staining of the cartilage matrix. MT compartment of a 4 month old (C) guinea pig shows no staining in the superficial zone devoid of chondrocytes, however in contrast, arrows indicate the staining of the fibrillated cartilage in the 7-month-old section (E). Arrows indicate the intracellular staining of chondrocytes throughout the deep zone of the articular cartilage. MT compartment of a 2-month-old guinea pig knee stained with secondary antibody only (negative control) (D). No extracellular staining of the epiphyseal plate was evident (F). Magnification bars: (A–E; 40× = 50 μM and F; 10× = 100 μM).

Coll2-1 was lowest at 3 weeks of age, increased by 65% from 3 weeks to 4 months of age ($P = 0.002$), and remained consistently elevated thereafter to 18 months of age. Levels of Coll2-1NO_2, the nitrosylated version of Coll2-1, were measured in the same samples and concentrations equaled 1–2% of the serum concentrations of Coll2-1 throughout the time course. The serum profile for Coll2-1NO_2 was similar to that of Coll2-1 and the two measures were highly correlated ($r = 0.9441$, $P < 0.0001$) (data not shown). Serum C2C decreased slightly from 3 weeks to 2 months of age, but there was no variation in mean C2C concentrations coincident with the development of histological OA. At 3 weeks of age, mean serum CTX-II was at its highest and rapidly declined to barely detectable levels by 7 months of age, showing very little change from 7 to 18 months of age. Correlations of the biomarkers were explored to assess the possibility that these serum biomarkers are reflective of inter-related biological processes. There was a significant positive correlation between Coll2-1 and C2C ($r = 0.3788$, $P = 0.009$). There was a significant negative correlation between CTX-II and Coll2-1 ($r = -0.4076$, $P = 0.005$). There was no significant correlation between CTX-II and C2C ($r = -0.1710$, $P = 0.2613$).

The total histological scores were divided into quartiles (Q) with the following ranges of scores (Q1: 0–0; Q2: 0–9; Q3: 10–18; Q4: 19–32). Mean levels of serum Coll2-1 increased significantly between Q1 and Q2 histology score ($P < 0.01$) and mean levels remained elevated and unchanged for the higher severity quartiles; Q1–Q3 ($P < 0.05$), Q1–Q4 ($P < 0.001$) (Fig. 8). There were no

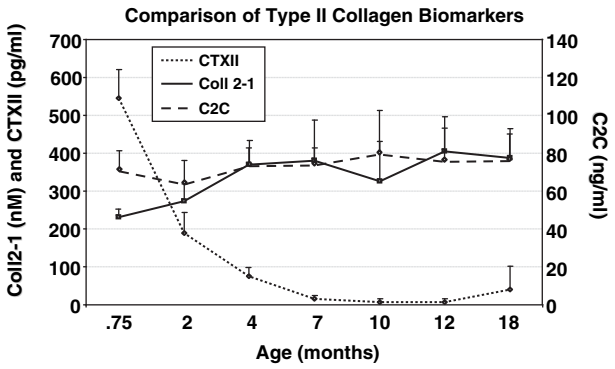

Fig. 7. Comparison of type II collagen degradation biomarkers. Measurement of type II collagen degradation biomarkers C2C, Coll2-1, and CTX-II in guinea pig sera from animals aged 3 weeks to 18 months. Results were expressed as mean ± SD.

significant differences in levels of serum C2C with onset and progression of histological OA. Levels of serum CTX-II were elevated in Q1 (young animals with no histological OA), and decreased significantly (ANOVA $P = 0.0001$) for higher severity quartiles, representing increasing severity of histological OA.

Discussion

In the present study, we observed reductions in normal collagen network birefringence beginning at 2 months of age, prior to evidence of histological OA and provide semi-quantitative characterization of the temporal course of this collagen fibril disruption. We conclude that articular collagen fibril disruption is an early OA-related event in this model system. Other means of analysis, namely magnetic resonance imaging (MRI), also point to this conclusion; MRI has provided evidence for cartilage swelling between 2 and 6 months of age in the Hartley guinea pig knee[26]. In studies of human tissue, swelling has been convincingly attributed to disruption of collagen[27], enabling the proteoglycan to absorb water. This provides further evidence in support of early collagen fibril disruption in this model system.

Several other studies have demonstrated alterations in collagen fibril integrity prior to disruption in the articular surface (one canine study[28], one rabbit study[19], and one guinea pig study[17]). In the guinea pig study, Han *et al.* observed a loss of normal birefringence of the articular cartilage in the knee and ankle joints with age and reported greater reductions on the tibial side of the joint compared with the corresponding femoral surface[17].

Previously, we demonstrated the presence of collagenase 1 and collagenase 3 in the extracellular matrix of Hartley guinea pigs as early as 2 months of age[3]. In this study, we have confirmed the expression of collagenase activity in sections as early as 2 months of age in both the medial and lateral compartments of the knee, prior to histological evidence of OA but coincident with collagen fibril disruption by birefringence. Although, the collagen abnormalities are widespread, the histological abnormalities of cartilage structure and proteoglycan content are differentially located in the medial compartment. Thus, it would appear that collagen cleavage and network disorganization is a necessary, but not sufficient cause for the histological manifestation of disease. It is likely that the greater mechanical load on the medial compartment, in conjunction with obesity, for which the guinea pig is well known, combine to accelerate the disease in the medial compartment in the face of collagen fibril disruption. The equivalent loss of birefringence in both medial and lateral compartments could reflect the overall metabolically active bone and cartilage which has been reported previously in the Hartley strain of guinea pig[14] and may provide an explanation for the prevalence of OA in this strain.

Both Coll2-1 and C2C displayed inverse serum profiles to that of serum CTX-II. The CTX-II epitope can result from the degradation of type II collagen in a single joint, as measured in synovial fluid[29], or may be reflective of degradation of type II collagen in mineralized tissue[30]. Serum levels of CTX-II reflected a pattern most compatible with collagen II turnover in the growth plate cartilage, being highest at 3 weeks of age and then rapidly declining by 4 months and barely detectable by 7 months of age onward. The growth plates in the guinea pig long bones do not ossify until 18–24 months, but bone growth, and by inference growth plate turnover, ceases at 4 months of age[31], and is barely measurable during the period of histological OA development in this model system.

Coll2-1 has been shown to be significantly elevated in OA patients compared to controls[24]. Levels of serum and urinary Coll2-1 have been shown to predict progression of radiographic knee joint space narrowing over 1 year in

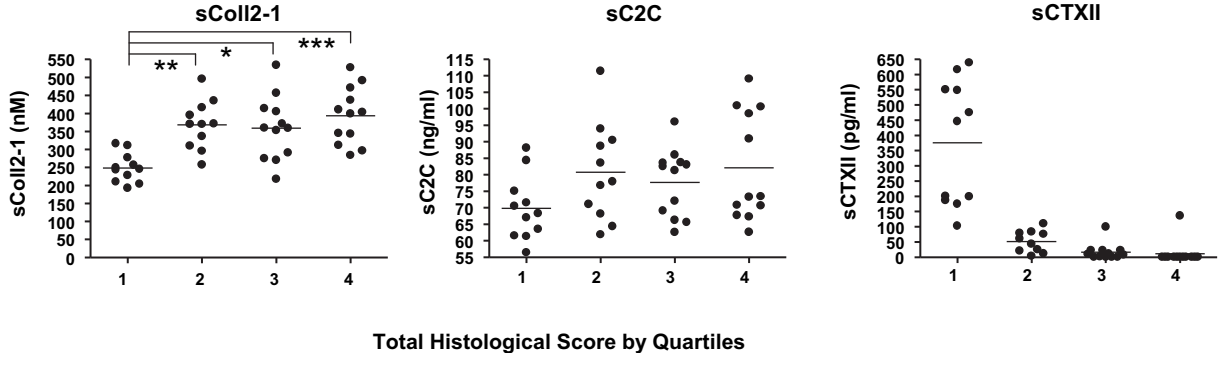

Fig. 8. Distribution of serum biomarker levels by quartiles of total histological score. Total histological scores were divided into quartiles (Q1: 0–0; Q2: 0–9; Q3: 10–18; Q4: 19–32) to examine the distribution of levels of serum biomarker by severity of histological OA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

humans[24,32], and to decline significantly following knee or hip joint replacement providing evidence for a joint specific origin of this marker[33]. The results in this study suggest that the Coll2-1 biomarker may be an early indicator of the onset of chondropathy in the guinea pig and an indicator of ongoing disease activity during the period of disease progression. Based on these results, we would categorize Coll2-1 as a burden of disease activity marker according to the nomenclature proposed by Bauer *et al.*[34]. It is both remarkable and fortuitous that growth plate collagen II turnover is not reflected in the serum levels of Coll2-1. In addition, the Coll2-1 epitope, in contrast to the 9A4 epitope, was not observed in the growth plate itself. The reason for this is unknown; however, it suggests that this marker may be more indicative of pathological articular cartilage type II collagen turnover. We speculate that the enzymes responsible for growth plate collagen turnover are different from those of pathological articular cartilage turnover and lead to different end biomarker products[12].

We were limited in this study by our inability to quantify levels of collagen biomarkers in synovial fluid, a site more proximal to the events under study. A previous report of CTX-II measurements in the synovial fluid of the rat required a larger volume than was available from our study. We also did not measure specific markers of proteoglycan turnover, as they were not available for this animal model. Such a biomarker may have provided a more sensitive measure of disease onset than was provided by histological evidence of proteoglycan loss.

Another limitation of this study was the lack of control animals which would have allowed for distinctions to be made between age-related and pathological changes in this model. However, our previous work, using Strain 13 guinea pigs as age-matched controls to the Hartley guinea pigs, demonstrated significant differences in cartilage and bone metabolism as well as susceptibility to OA, distinguishing the OA process from an age-related phenomenon[14]. These findings were later confirmed by subsequent studies using other strains of guinea pig as age-matched controls[16,35–39]. Together, these studies support the hypothesis that changes occurring in the Hartley strain guinea pig are not likely to be simply due strictly to age, but more specifically to pathological alterations in cartilage, bone, metabolism, ligaments, and biomarkers that make this strain more susceptible to the development of OA.

In summary, disruption of the collagen network integrity appears to be a very early event in the cascade of joint degenerative processes in the Hartley guinea pig model of knee OA, as evidenced by the time course of the loss of birefringence, creation of neoepitopes specific to cleavage by collagenases, and serum type II collagen biomarkers. We speculate that disruption of the collagen network predisposes to OA and, in conjunction with excess mechanical load localized to the medial compartment and exacerbated by obesity, leads preferentially to progression of histological OA in the MT compartment.

Conflict of interest

Janet Huebner, James M Williams and Virginia Byers Kraus have no conflict of interest related to this work. Michelle Deberg and Yves Henrotin receive royalty payments from Zentech for their commercialization of Coll2-1. All Coll2-1 measurements by Drs. Deberg and Henrotin were made blinded to animal status and the statistical analysis of all data was performed at a separate site (Duke).

Acknowledgments

Supported in part by the NIH/NIAMS (AG15108 and AR50245). We wish to thank Susan Reeves for her expertise and assistance in the preparation of figures containing the photomicrographs.

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